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PATENT
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Applicants: Biedermann et al.

Serial No.: 09/242,540

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Title: PYRIDYL ALKENE AND PYRIDYL ALKINE ACID AMIDES AS CYTOSTATICS AND IMMUNOSUPPRESSIVES

Group Art Unit: 1624

Examiner: Coleman

DECLARATION OF HASMANN UNDER 37 CFR 1.132

Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

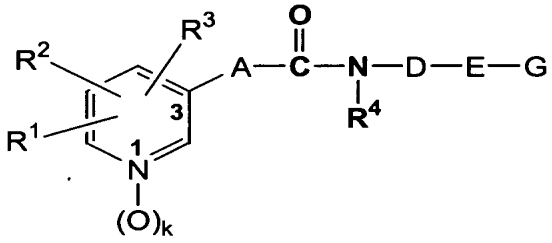
Dear Sir:

I, Max Hasmann, pursuant to 37 C.F.R. §1.132, declare as follows:

1. I am one of the inventors for the above-identified patent application.

2. Compounds of the present invention are represented by the general formula set forth below. Under my supervision experiments have been conducted to demonstrate the importance of the substitution of the pyridine ring in its 3-position (shown in the structure below in bold), the criticality of "A", and the importance of having an amide

group in the chain of the substituent attached to the 3 position of the pyridine ring (shown in the structure below in bold).



3. Tumor growth inhibiting activity of test compounds was determined on human tumor cells in standard in vitro test systems. The three types of cell lines used in testing was as follows.

Cell Line	Origin
Hep G2	liver carcinoma
THP-1	monocytic leukemia
A549	lung carcinoma

As further described below, the screening tests were used to determine an IC_{50} value (defined as the concentration in which the cell growth was inhibited by 50%) of each test compound. A low IC_{50} value indicates that the test compound has a high tumor growth inhibiting activity, whereas a high IC_{50} value, for example an IC_{50} value greater than 10 μM, indicates that the test compound has a low tumor growth inhibiting activity.

4. Tests with Hep G2 cells:

Hep G2 cells derived from a human liver carcinoma were plated at a density of 20,000 cells/ml in 12-well plastic dishes. Cultivation occurred in Richter's IMEM-ZO nutrient medium with 5% fetal calf serum (FCS) in a tissue culture incubator with a gas mixture of 5% CO_2 and 95% air at a temperature of 37°C. One day after plating, the culture medium was aspirated from the cells and replaced by fresh medium which contained the respective concentrations of the test substances. For the individual concentrations and the controls without test substances, three-fold batches were done for each. Three days after the beginning of treatment, the medium was again renewed with the test compounds. After six days of substance incubation, the test was ended and the

protein amount in the individual wells was determined with the sulforhodamine-B-method (according to *P. Skehan et al.: New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening, J. Natl. Cancer Inst. 82: 1107-1112, 1990*). The IC_{50} values were taken from the dose-response curves and given as a comparative measurement for the activity of the test compounds.

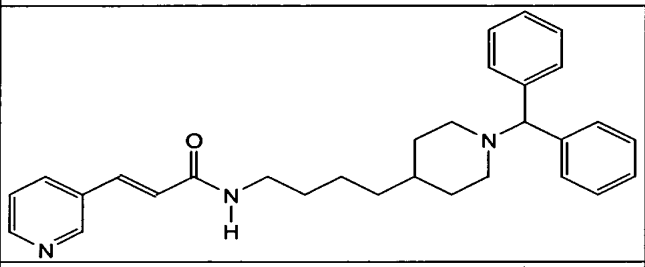
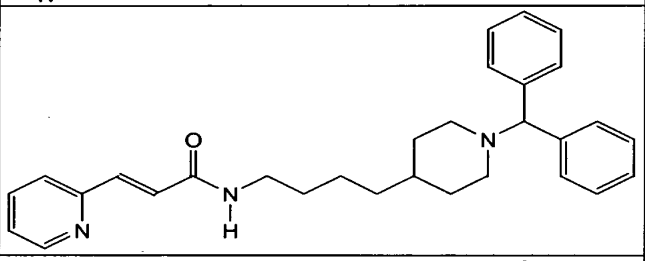
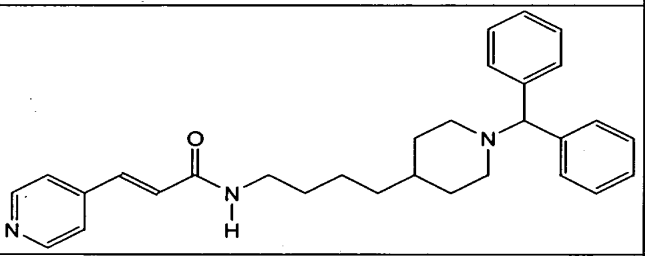
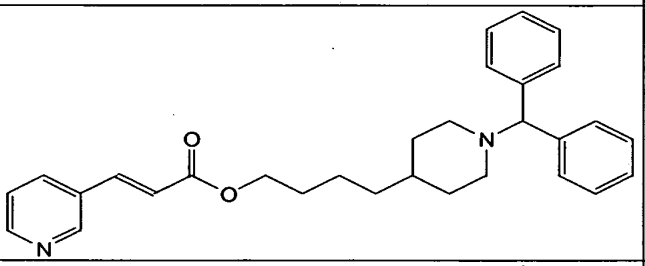
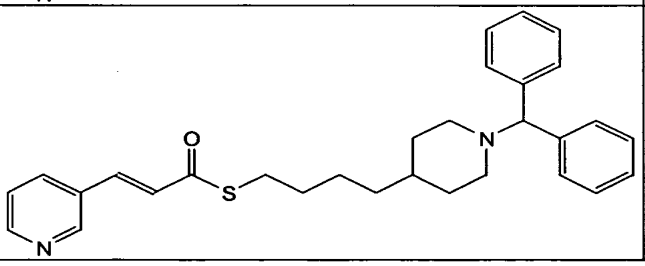
5. Tests with THP-1 cells:

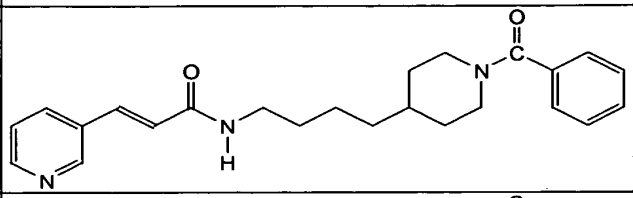
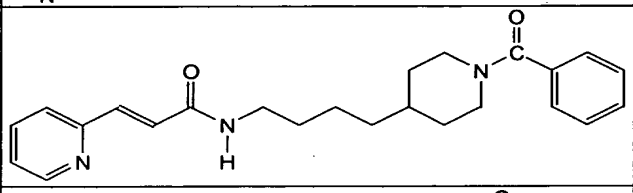
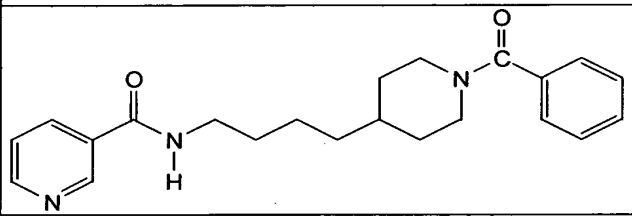
THP-1 cells derived from a human monocytic leukemia were plated at a density of 200,000 cells/ml in 96-well flat-bottom microtiter plates. Cultivation occurred in RPMI 1640 nutrient medium with 10% fetal calf serum (FCS) in a tissue culture incubator with a gas mixture of 5% CO_2 and 95% air at a temperature of 37°C. For the individual concentrations and the controls without test substances as well as for the background with nutrient medium but without cells, three-fold batches were done for each. After four days of substance incubation 20 µl of WST-1 reagent (Boehringer Mannheim) were pipetted in each individual well. After 30 to 60 minutes of incubation in the tissue culture incubator at 37°C and 5% CO_2, the light extinction was measured in an ELISA reader at a wave length of 450 nm. The background values were subtracted from the measured values. The IC_{50} values were taken from the dose-response curves and given as a comparative measurement for the activity of the test compounds.

6. Tests with A549 cells: A 549 were screened according to the procedure described in paragraph 4. In order to encounter the higher proliferation rate of A549 cells the treatment with test substances was terminated after 4 days of incubation.

7. Screening Results:

Compound having IC_{50} values > 10 μM showed a low tumor growth inhibiting activity which is expected by a person skilled in the art not to be applicable in vivo due to solubility problems and/or potential toxicity of high dosages.

No.	Compound	IC_{50} Value [μM]			Remarks
		HepG2	THP-1	A549	
312		0.001	0.008	0.04	3 position, amide group (invention)
312.A2		>10	n.d.	>10	no 3 position
312.A4		>10	n.d.	>10	no 3 position
312.D1		>10	n.d.	>10	ester instead of amide group
312.D2		>10	n.d.	>10	thioester instead of amide group

No.	Compound	IC_{50} Value [μM]			Remarks
		HepG2	THP-1	A549	
159		0.0004	0.0002	0.002	"A" present, 3 position (invention)
159.A2		>10	n.d.	>10	no 3 position
159.C		n.d.	n.d.	>10	no "A" present

n.d.: not determined

8. The test results set forth above demonstrated that the substitution of the pyridine ring in its 3-position, having an "A" rather than only a bond, and the amide group in the substituent attached to the 3-position of the pyridine ring are extremely important for tumor inhibiting activity.

As shown by the data set forth above, compounds with a pyridine ring substituted in the 2- and in the 4-position did not show tumor inhibiting activity in concentrations usable in vivo.

Compounds having bond instead of "A" did not inhibit tumor growth in concentrations usable in vivo.

Further, as shown by the data set forth above, compounds where the amide in the substituent attached to the 3-position of the pyridine ring are replaced with ester or thioester did not show tumor inhibiting activity in concentrations usable in vivo.

The undersigned, being warned that wilful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. §1001) and may jeopardize the validity of the application or any patent issuing thereon, hereby declares that the above statements made of my own knowledge are true and that all statements made on information and belief are believed to be true.

Date: Nov. 7, 2001

Max Hasmann